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Research Article

Erythrina Indica Ethyl Acetate Extract Inhibits Diethyl Nitrosamine-Induced Developmental Toxicity via Changing the Notch Signalling Pathway in Zebrafish Embryos

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Abstract - Diethyl nitrosamine (DEN), a potent liver carcinogen derived from secondary amines found in fertilizers and pesticides, has garnered attention due to its widespread presence in tiny amounts in food and water. It has the potential to induce cancer of the liver and digestive tract in living organisms. The tree Erythrina indica, also known as E. indica, possesses antioxidant and anti-inflammatory properties, and it may possibly have hepatoprotective effects. Because of their short reproductive cycles and translucent embryos, which make it easy to detect developmental toxicity, zebrafish embryos are ideal for creating pre-regulatory medications. We used the fish's embryos for this experiment. In addition to DEN, some of the embryos also got E. indica leaf extracts. By comparing the development of embryos exposed to DEN with those that had further treatment with E. indica leaves, we were able to demonstrate a possible protective effect against this carcinogen by lowering the toxicity of DEN in these embryos.

Keywords - *Diethyl nitrosamine, Developmental toxicity, Zebrafish embryos.*

I. INTRODUCTION

A. Erythrina Indica

Native to eastern Africa, the Indian subcontinent, northern Australia, and the islands in the western and Indian Pacifics, east of Fiji, is the Erythrina species. Erythrina indica trees are medium-sized and growing rapidly; they are found in Bengal and all over India, especially in southern India. It is also referred to as the Indian coral tree (in English) and the Kalyana murungai (in Tamil). This deciduous tree, which can reach a height of 27 meters (89 feet), is prickly and produces dense racemes of flowers.

Figure 1. Erythrina indica

i). Uses It's a plant with numerous medicinal applications and health benefits. Traditional medicine has made use of the plant's different parts because they are thought to have analgesic, diuretic, antioxidant, anti-inflammatory,

and anticonvulsant properties. Leaf extract is applied as a toothache and ear anodyne. To ease discomfort in rheumatoid joints, crushed leaves are applied. Its leaves help get rid of intestinal worms and are used to decrease blood pressure. Additionally, it is said to have hepatoprotective properties.

Antioxidant properties of stem bark from E. indica are purported. There are claims that the ethanolic extract from its bark has anti-inflammatory properties. The leaf extracts cause a decrease in blood sugar levels in us. It's also claimed to reduce cholesterol. The plant is reported to have several phenolic metabolites. The presence of glycosides, tannins, saponins, alkaloids, flavonoids, and phytosterols was discovered during a previous phytochemical analysis.

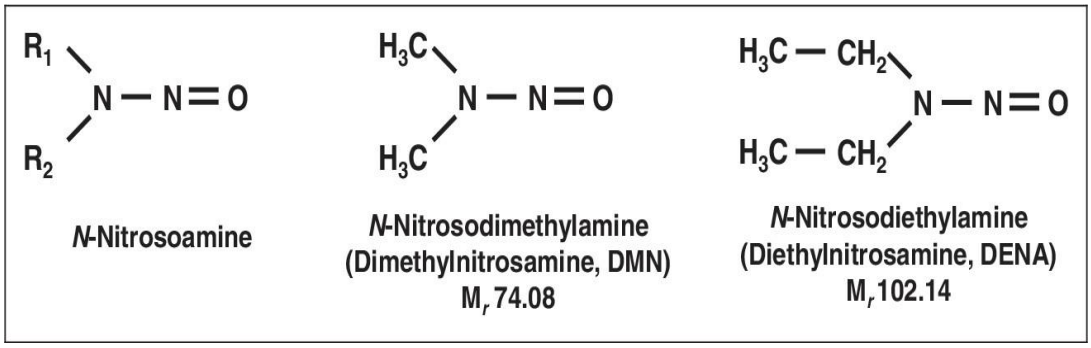

Figure 2. Structures of N-Nitrosamine

B. Diethylnitrosamine

It is well recognized that nitrosamines have a high carcinogenic potential. Certain sources claim that nitrosamine intoxication causes cancer of the liver and digestive tract. They are found in herbicides and fertilizers. Diethylnitrosamine (DEN) is the most common type of nitrosamine. Owing to the extensive use of fertilizers and pesticides in modern times, DEN is present in a variety of foods, such as cheese, smoked meat, dried fish, and water. In addition, they can be found in infant bottle nipples and tobacco. DEN disrupts the processes responsible for DNA replication and repair, leading to mutation. It is a strong hepatocarcinogen that is utilized in scientific settings to research cancer in rodent models.

C. Notch Signaling Pathway

A limited set of signaling channels are employed repeatedly during development to regulate cell fates, proliferation, and death. The Notch receptor, which is distinct in that most of its ligands are also transmembrane proteins, is involved in one such route. Signaling is therefore restricted to cells that are next to one another. The Notch signaling system is a highly conserved cell signaling mechanism seen in most mammals.

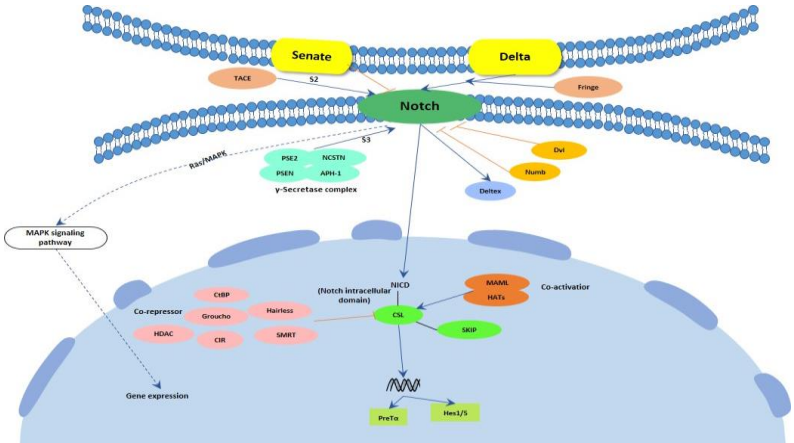

Figure 3. Notch Signal Pathway

D. Zebrafish Embryo

In contrast to the more costly and labor-intensive use of mammals, zebrafish which were first employed as a model for studying development and neurobiology approximately thirty years ago, have grown in value due to their tiny size, cheap maintenance costs, and quick development. Up to 10,000 eggs can be laid by afemale

zebrafish each year, and the embryos grow swiftly. Around 10 hours after fertilization (hpf), the gastrulation stage concludes, and somites are formed during the segmentation stage. Somatogenic development is finished, and different organ rudiments are created by 24 hpf. By 28 to 30 hours post fertilization, the embryos are mobile and exhibit their first behavior as a startle response. They start feeding five days after fertilization (dpf), a sign that most organs have reached functional maturity. Thanks to technical developments, the zebrafish embryo is now a crucial component of drug-screening and disease modeling systems, especially during the pre- clinical the later stages of drug development, rodents cannot be completely replaced by early larval and zebrafish embryos, although they are good screening tools that complement rather than completely replace rat or cell-based studies.

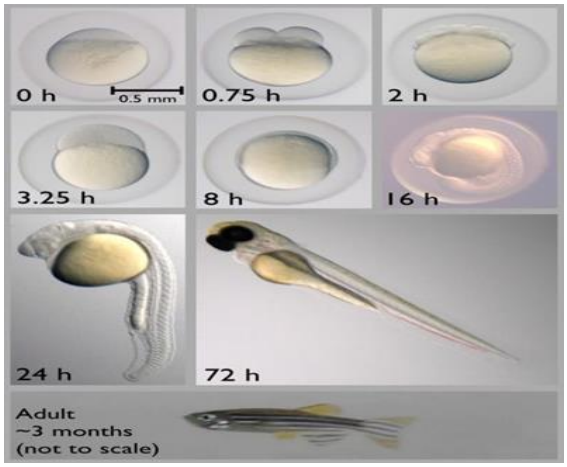

Figure 4. Zebrafish Embryo

II. REVIEW OF LITERATURE

R. Tolba, T. Kraus, C. Liedtke, M. Schwarz, and R. Weiskirchen (2015) stated that nitrosamines, including DEN, are acute hepatotoxins and carcinogens in numerous mammal species. Long-term oral feeding or parenteral injection of high doses of DEN is an effective way to cause liver tumors in mice.

Amy L. Rubinstein (2003) noted that there is mounting evidence that zebrafish accurately imitate human biology and that pharmaceuticals that are already being used in humans can have predictable effects in zebrafish assays. Because mammalian illness models are expensive and often not suitable for high-throughput target validation and drug screening, the zebrafish system may offer a crucial link between high-throughput in vitro experiments and in vivo models of disease.

Yang, L., Ho, N According to Strähle, U. (2009), using zebrafish embryos as a model for the embryotoxic and teratological effects of chemicals during the early stages of drug development could result in a significant reduction in the cost of new medications and provide a more ethically sound alternative to current testing.

S., Champagne, D. L., Spaink, H. P., & Richardson, M. K. (2011) state that it can be argued that drug therapies that are effective in rodent and zebrafish models will have the best chance of being effective in treating human patients.

The zebrafish embryo is crucial for understanding the mechanisms of developmental toxicity in developmental biology, according to Teraoka, H., Dong, W., & Hiraga, T. (2003) in their study.

Shi, X., Du, Y., Lam, P. K. S., Wu, R. S. S., & Zhou, B. (2008) stated that increased juvenile malformations, decreased hatchability, altered heart rate and cardiac malformations, and impaired juvenile viability are indicators. All of these are typical stress reactions in embryos exposed to toxins.

In their paper "Developmental Toxicity of Diethylnitrosamine in Zebrafish Embryos/Juveniles Related to Excessive Oxidative Stress," Huang, D., Li, H., He, Q., Yuan, W., Chen, Z., & Yang, H. (2018) reported that zebrafish embryos exposed to DEN showed a range of abnormalities, including growth retardation, notochord malformation, pericardial and variable levels of death. Cell-cell interactions mediated by the Notch signaling pathway occur throughout C. elegans embryogenesis, according to James R. Priess' paper from 2005, These relationships are essential for determining cell fates and tissue morphogenesis.

Weinmaster, G., & Kintner, C. (2003) state that the Notch signaling system mediates regional cell-cell interactions, which frequently regulate cell fate, and thus controls distinct facets of tissue differentiation during embryonic development. A core Notch signaling pathway has been biochemically identified because the conserved components underlying Notch signaling have been isolated in vertebrates, and functional studies of this system during embryogenesis have been carried out.

In their study from 2017, "Evaluation of hepatoprotective potential of Erythrina indica leaves against antitubercular drugs induced hepatotoxicity in experimental rats," The methanolic extract of E. indica demonstrated dose-dependent influence in reducing the liver damage induced by INH-RIF in the rats, according to study results by Mujahid, M., Hussain, T., Siddiqui, H. W., & Hussain, A. The properties are hepatoprotective.

III. MATERIALS AND METHODS

A. Extraction

i). Preparation of Plant Sample

Erythrina indica leaves were gathered from the Tamil Nadu district of Kanchipuram, cleaned, and subsequently dried at the ambient temperature. Using a mixer grinder, the dried leaves were ground into a fine powder. Assessing the powdered leaves, we sealed these into a hermetic bag.

ii). Solvent Extraction

Twenty milligrams of E. indica leaf powder have been added to a clean, conical flask. The conical flask was then filled with 200 ml of the ethyl acetate solvent. Once covering the flask wit silver foil, it was left alone for a period of three days. After that, the Whatman No. 1 filter paper was used to filter the extract.

Figure 5. Filtration

B. Total Antioxidant Capacity (TAC) Assay

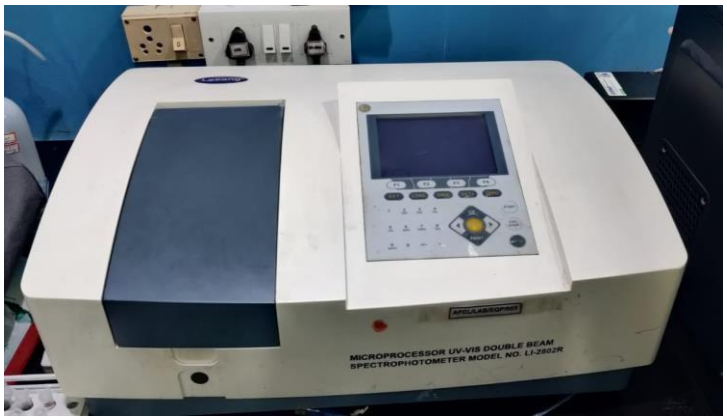

Figure 6. UV – Visible Spectrophotometer

i). Procedure

Dimethyl sulfoxide (DMSO) was used to dissolve 30 mg of plant extract to create the extract's stock solution. Different volumes of the extract (100, 200, 300, 400, 500) µl were applied to test tubes S1, S2, S3, S4, and S5, respectively, while the control (B) was left with water. After that, 1.5 milliliters of the test reagent for total antioxidant capacity were applied. After thorough mixing, the test tubes were held at 95°C for ninety minutes. Using a UV-Vis spectrophotometer, the absorbance at 695 nm was measured following the incubation. The % inhibition was computed using the obtained absorbance values, and the graph was plotted using the resulting formula.

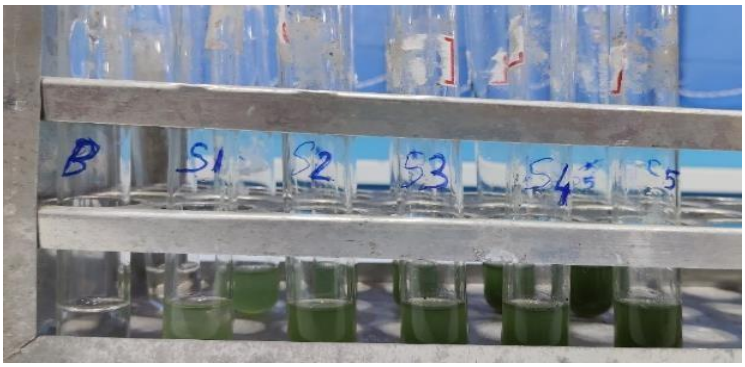

Figure 7. TAC Assay Carried OUT in Test Tubes

% Inhibition = ((A0-A1) / A0 × 100)
Were,
A0 = Control OD
A1 = Sample OD

C. Human RBC Membrane Lysis Assay

This test is anti-inflammatory. Given that the membranes of human red blood cells (HRBCs) and lysosomal membrane components are comparable, the capacity of different extracts to prevent hypotonicity-induced HRBC membrane lysis was employed as a gauge for their anti-inflammatory potential.

i). Procedure

10 mg of plant extract and 1 ml of ethanol were dissolved to create the extract's stock solution. Plant extracts in varying amounts (10, 20, 30, 40, and 50) µl were added to the Eppendorf tubes S1, S2, S3, S4, and S5, and increased to 1 ml by adding distilled water. whereas 1 milliliter of saline was administered to the control (B).

D. Dosage Fixation of Drug

i). Procedure

Ten milligrams of plant extract were dissolved in one milliliter of ethanol to create the extract's stock solution (10 mg/ml). Six sterile glass bottles were then taken; they were labeled control, 100µg, 200µg, 300µg, 400µg, and 500µg. Each bottle was then filled with 200ml of oxygenated water. The 100µg, 200µg, 300µg, 400µg, and 500µg bottles received 10µl, 20µl, 30µl, 40µl, and 50µl of the extract (drug), respectively. No extract was given to the control container.

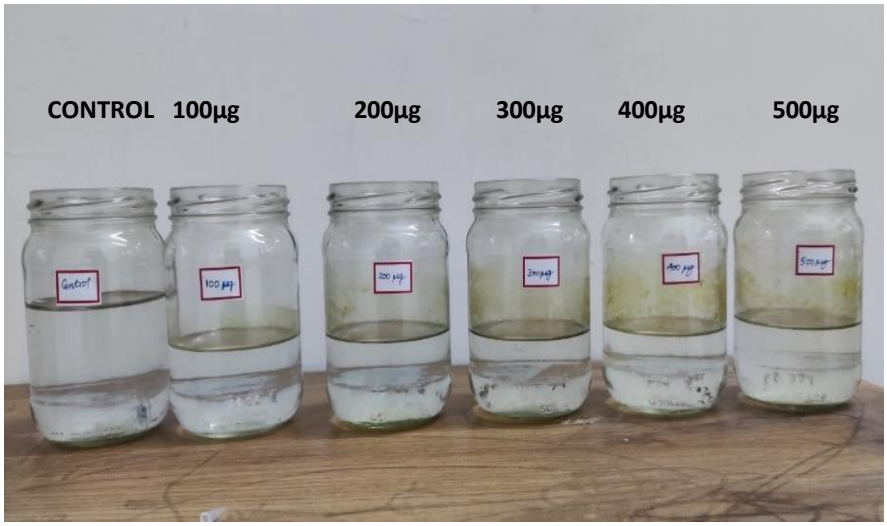

Figure 8. Zebrafish Embryos Kept in Glass Bottles

On the second day, fresh water was put to the bottles along with the extract. After the water was withdrawn on Day 3, the embryos were collected and sacrificed into several Eppendorf tubes that contained Tris-HCl. To conduct more tests, the Eppendorf tubes were kept in a deep freezer.

E. Protein Isolation

The embryos inside the Eppendorf tubes were removed and allowed to defrost. Using a mortar and pestle, the embryos were crushed before being centrifuged for ten minutes at 12,000 rpm at 4°C. After being collected, the supernatant was kept in a freezer.

F. Protein Isolation

i). Procedure

In Eppendorf tubes, 50 µl of protein derived from 100 µg, 200 µg, 300 µg, 400 µg, and 500 µg was collected. Each tube received 500µl of the Bradford reagent. For thirty minutes, the tubes were incubated at room temperature. Using a UV-visible spectrophotometer, the absorbance value at 595 nm was determined. Plotting the graph required applying the following calculation to determine the protein concentration based on the observed absorbance values.

Protein concentration = (Sample OD / Standard OD) * Standard concentration

G. Lactate Dehydrogenase Assay

i). Procedure

One milliliter of lithium lactate and the protein extracted from 50 µl of embryos were put into the test tube. For fifteen minutes, the solution was incubated at 37°C. Each tube was then filled with 1 milliliter of DNPH and incubated for an additional 15 minutes at 37°C. Each tube received 7 ml of NaOH after the incubation period. At 420 nm, the absorbance values were measured. The formula for calculating Ldh activity is as follows.

LDH (U/L) = (A420 nm X total volume) / (6.22 X 103 X sample volume X path length)

H. Lactate Dehydrogenase Assay

i). Protein Isolation

The embryos inside the Eppendorf tubes were removed and allowed to defrost. Using a mortar and pestle, the embryos were crushed before being centrifuged for ten minutes at 12,000 rpm at 4°C. After being collected, the supernatant was kept in a freezer.

ii). Procedure

After being removed from the deep freezer, the separated proteins were thawed. Six Eppendorf tubes have been labeled as control, induction, and treatment, along with duplicates of each. 50 µl of protein from the treatment, induced, and control groups was added to each tube in turn. Then, each tube received 500 µl of Bradford reagent. 30 minutes of room temperature incubation produced the absorbance values at 595 nm. The provided formula was used to estimate the protein content.

Protein concentration = (Sample OD / Standard OD) * Standard concentration

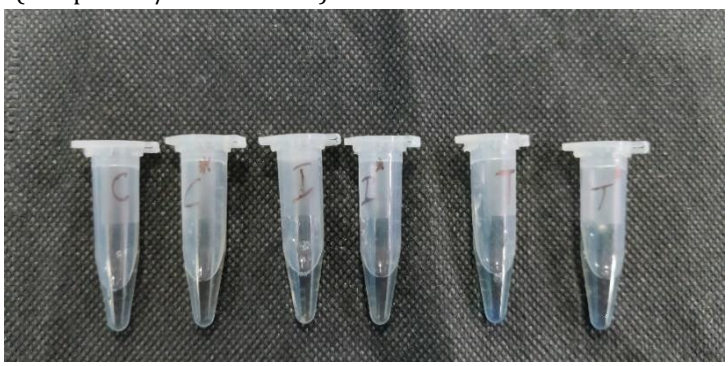

Figure 9. Zebrafish Embryos Kept in Glass Bottles

I. Lactate Dehydrogenase Assay

i). Procedure

After being removed from the deep freezer, the separated proteins were thawed. Three test tubes have been taken and labeled as treatment, induction, and control. 50µl of the protein from the treatment, induced, and control groups was added to each tube in turn. Each tube was then filled with 1 milliliter of lithium lactate. For fifteen minutes, the solution was incubated at 37°C. Each tube was then filled with 1 milliliter of DNPH and incubated for an additional 15 minutes at 37°C. Each tube received 7 ml of NaOH after the incubation period. At 420 nm, the absorbance values were measured. The formula for calculating Ldh activity is as follows.

LDH (U/L) = (A420 nm X total volume) / (6.22 X 103 X sample volume X path length)

Protein samples, Lithium lactate, DNPH (2,4 – Dinitrophenylhydrazine), NaOH (sodium hydroxide), UV – Vis Spectrophotometer. The embryos inside the Eppendorf tubes were removed and allowed to defrost. Using a mortar and pestle, the embryos were crushed before being centrifuged for ten minutes at 12,000 rpm at 4°C. After being collected, the supernatant was kept in a freezer.

J. Griess Assay

i). Procedure

After being removed from the deep freezer, the separated proteins were thawed. Six Eppendorf tubes have been labeled as control, induction, and treatment, along with duplicates of each. 50µl of the protein from the treatment, induced, and control groups was added to each tube in turn. After that, each tube received 500µl of Griess reagent, and it was incubated for 30 minutes at night. The absorbance readings were measured at 540 nm following incubation. Using the following formula, the griess assay's activity was calculated:

y (OD value) = 0.008x – 0.003

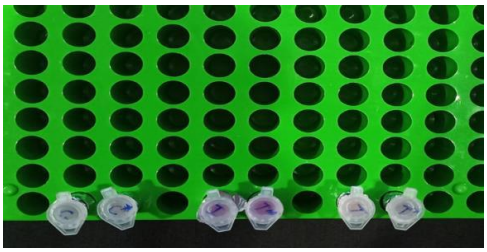

Figure 10. Eppendorf Tubes Placed in Stand

IV. RESULTS

A. Extraction

The Extraction was performed twice where the final weight of extract obtained were:

First extract– 0.9 g

Second extract – 0.5 g

B. Phytochemical Assays

The Salkowski test, which looks for sterols and terpenoids, the Ammonium test, which looks for flavonoids, and the Ferric chloride test, which looks for tannins, were all positive, according to the above table.

Table 1. Phytochemical Assay Result

S.NO	TESTS	BLANK	EXTRACT
1.	SALKOWSKI TEST	-	+
2.	AMMONIUM TEST	-	+
3.	ALUMINIUM CHLORIDE TEST	-	-
4.	FERRIC CHLORIDE TEST	-	+
5.	PHENOL TEST	-	-
6.	KILLER KILLANI TEST	-	-
7.	BIURET TEST	-	-
8.	WAGNER'S TEST	-	-
9.	NINHYDRIN TEST	-	-
10	FEHLING'S TEST	-	-

Figure 11. Salkowski Test

Figure 12. Ammonium Test

Figure 13. Ferric Chloride Test

C. Phytochemical Assays

Table. 2 Absorbance Values and TAC Activity Values of TAC Assay

VOLUME OF EXTRACT (µl)	OD at 695 nm	TAC ACTIVITY
100	0.532	40.93
200	0.616	31.61
300	0.606	32.72
400	0.625	30.61
500	0.654	27.39

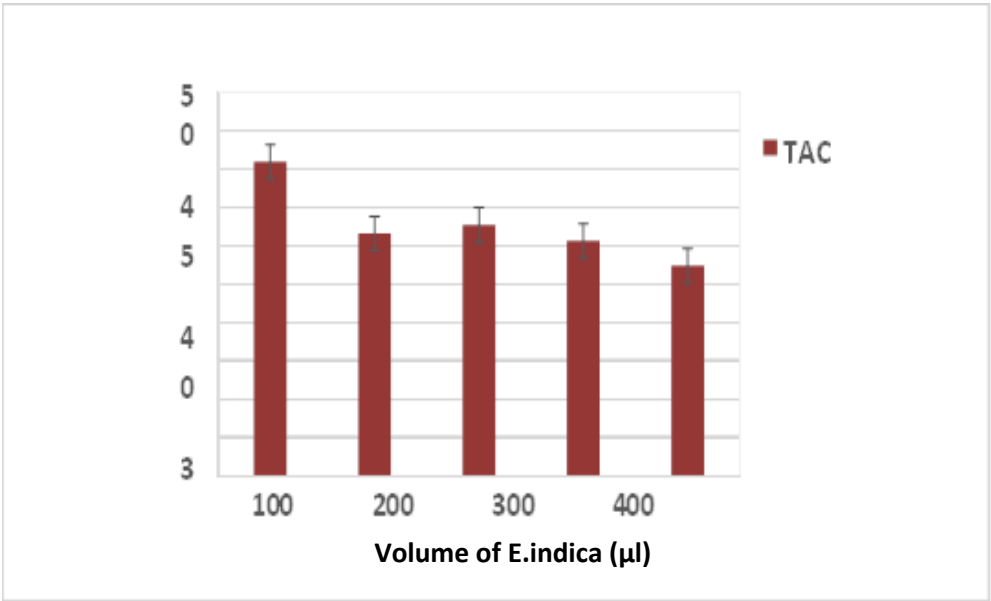

Figure 14. Graphical Representation of Volume of Extract vs TAC Activity

D. Histopathology Study

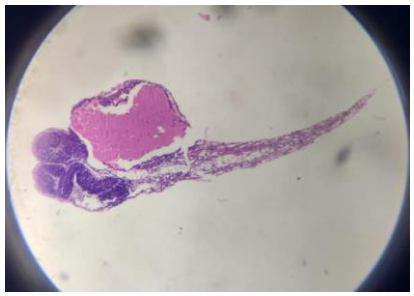

Figure 15. Control Viewed Under Microscope

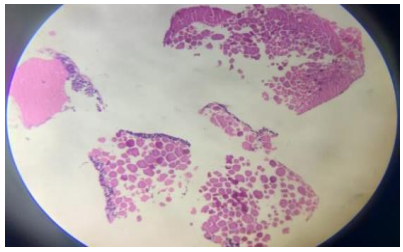

Figure 16. Induction Viewed Under Microscope

V. GENERAL DISCUSSIONS

One of the most prevalent forms of N-nitrosamine, a strong liver carcinogen, is diethylnitrosamine. These days, DEN can be discovered in food goods, agricultural products, and water due to the widespread use of fertilizers and pesticides that contain N-nitrosamine. Due to its carcinogenic properties, this DEN can induce liver and digestive tract cancer. It is believed that DEN metabolites cause DNA damage, which can result in mutations, cell death, and decreased cell proliferation and differentiation. The exact processes underpinning DEN-induced developmental toxicity are yet unknown.

Furthermore, exposure to DEN can result in inflammation, oxidative stress, and altered gene expression, all of which can exacerbate developmental problems. According to reports, zebrafish embryos are translucent, making it simple for us to see developmental toxicity in them.We think that pre-regulatory stages of drug development, particularly the initial stages, can benefit from the use of zebrafish embryo screens. Negative consequences on embryo development, including notochord abnormalities, tail hypoplasia, growth deformity, and egg coagulation, are attributed to DEN. There haven't been many reports on DEN's developmental toxicity in zebrafish embryos.

A. Tac Assay

Total antioxidant capacity (TAC), among is a measurement of the amount of free radicals scavenged by a test solution and is used to evaluate the antioxidant capacity of biological samples. The graph (figure 7) shows us that the extracts's antioxidant activity falls within a certain range, suggesting that varied extract concentrations are thought to have antioxidant qualities.

B. Human RBC Membrane Lysis Assay

Since these lysosomal membrane components are comparable to those of human red blood cells (HRBCs), the capacity of different extracts to inhibit lysis was employed as a gauge for their anti-inflammatory properties. Therefore, a method for determining the anti-inflammatory property has been used: the HRBC method. The graph (figure 8) shows that the percentage inhibition falls with increasing extract volume, indicating a reduction in the extract's antinflammatory properties. It is said that different extracts from the same plant have different properties. In 100µg, the anti-inflammatory property is strong.

VI. CONCLUSION

We were able to visualize the developmental toxicity of diethylnitrosamine with the aid of zebrafish embryos. The ethylacetate extract of Erythrina indica is claimed to have shielded the embryos against the toxicity of DEN, based on the assays and research conducted. The Notch signal pathway is thought to have been impacted by the DEN, causing the embryo to clot and negatively impacting its growth; in contrast, the E. indica extract is thought to protect the embryos undergoing therapy.

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